



# A major bristle QTL from a selected population of *Drosophila* uncovers the zinc-finger transcription factor Poils-au-dos, a repressor of *achaete–scute*

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Received for publication 14 July 2005, revised 12 September 2005, accepted 13 September 2005

Available online 10 October 2005

## Abstract

Traditional screens aiming at identifying genes regulating development have relied on mutagenesis. Here, we describe a new gene involved in bristle development, identified through the use of natural variation and selection. *Drosophila melanogaster* bears a pattern of 11 macrochaetes per heminotum. From a population initially sampled in Marrakech, a strain was selected for an increased number of thoracic macrochaetes. Using recombination and single nucleotide polymorphisms, the factor responsible was mapped to a single locus on the third chromosome, *poils au dos*, that encodes a zinc-finger-ZAD protein. The original, as well as new, presumed null, alleles of *poils au dos*, is associated with ectopic *achaete–scute* expression that results in the additional bristles. This suggests a possible role for Poils au dos as a repressor of *achaete* and *scute*. Ectopic expression appears to be independent of the activity of known *cis*-regulatory enhancer sequences at the *achaete–scute* complex that mediate activation at specific sites on the notum. The target sequences for Poils au dos activity were mapped to a 14 kb region around *scute*. In addition, we show that *pad* interacts synergistically with the repressor *hairy* and with Dpp signaling in posterior and anterior regions of the notum, respectively.

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**Keywords:** *Achaete–scute*; Bristle; Selection experiment; Mapping; *Drosophila*; Development

## Introduction

The large bristles (macrochaetes) on the notum of *Drosophila melanogaster* are arranged in a stereotyped array. Development of these bristles has been the focus of detailed genetic analysis (Calleja et al., 2002; Gomez-Skarmeta et al., 2003). Bristle development is dependent on the activity of basic Helix–Loop–Helix transcription factors encoded by the *achaete* (*ac*) and *scute* (*sc*) genes (Gomez-Skarmeta et al., 2003; Villares and Cabrera, 1987). The positioning of bristles is achieved through precise spatial regulation of *ac–sc* expres-

sion in small clusters of cells, the proneural clusters, at the sites of the future bristles in third larval instar wing imaginal discs (Cubas et al., 1991; Skeath and Carroll, 1991). Expression in proneural clusters is regulated by multiple, independently-acting *cis*-regulatory enhancer modules scattered throughout the 100 kb or so of the *ac–sc* complex (AS-C) (Gomez-Skarmeta et al., 1995; Ruiz-Gomez and Modolell, 1987). These mediate activation by transcription factors such as Pannier and Araucan/Caupolican (Garcia-Garcia et al., 1999; Gomez-Skarmeta et al., 1996; Leyns et al., 1996). Expression of *ac–sc* is then progressively refined within each cluster to the bristle precursors by autoregulation and Notch-mediated lateral inhibition (Culi and Modolell, 1998; Heitzler and Simpson, 1991; Kooh et al., 1993). The bristle pattern also relies on the activity of repressors such as Hairy and Extramacrochaetae, which prevent accumulation of Ac–Sc at positions where proneural clusters do not form (Ellis et al., 1990; Garrell and Modolell, 1990; Martinez et al., 1993; Van Doren et al., 1991, 1992, 1994).

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The pattern of bristles on the notum varies between Dipteran species and a few differences are even found within the genus *Drosophila* (Grimaldi, 1990; Simpson et al., 1999). Differences in the arrangements of bristles between species correlate with changes in the temporal–spatial expression patterns of *ac-sc* and at least one of their upstream regulators, *pannier* (Pistillo et al., 2002; Skaer et al., 2002; Wülbeck and Simpson, 2000, 2002). So there must exist sufficient variation in the genes regulating bristle patterning within a species to serve as a substrate for the evolution of bristle patterning between species (Skaer and Simpson, 2000).

Variation of bristle number in *Drosophila* has been a classical model for quantitative genetics. Quantitative trait locus (QTL) analysis of lines selected for an increased number of bristles, has shown that a small number of factors of large effect are usually involved. They often map close to genes with a known role in bristle development or more generally in nervous system development (Gurganus et al., 1998; Long et al., 1995; Norga et al., 2003). Naturally occurring variation at some of these loci such as the AS-C (Long et al., 2000), *scabrous* (Lyman et al., 1999), the *Delta-Hairless* region (Long et al., 1998; Lyman and Mackay, 1998) or *hairy* (Robin et al., 2002), contribute to quantitative variation in bristle number. Most quantitative genetic studies have focussed on the sternopleural and abdominal bristles, which are highly variable compared to the bristles found on the notum. Indeed, a “wild-type” pattern of 11 macrochaetes per heminotum is assumed to be fixed in *D. melanogaster*. However, natural variants of this pattern can be found and enough genetic variation exists in nature to select flies for an increased number of notal bristles (Dominguez et al., 1993; Macdowell, 1915; Pineiro, 1992a; Pineiro, 1992b; Plunkett, 1926; Sheldon and Milton, 1972). Selection experiments for ectopic dorso-central bristles have uncovered the influence of the genetic background and shown that the anterior and posterior dorso-central bristles can to some extent respond independently to selection (Dominguez et al., 1993; Macdowell, 1915; Pineiro, 1992a,b; Plunkett, 1926; Vreezen and Veldkamp, 1969).

In some cases, QTL do not map close to genes known for their role in bristle development (Dilda and Mackay, 2002; Nuzhdin et al., 1998). The study of natural variants can therefore lead to the discovery of new genes and give insights into bristle patterning mechanisms. Here, we present the analysis of a natural variant for the thoracic bristle pattern from a selected population of *D. melanogaster* initially sampled in Marrakech, Morocco, and uncover a new gene involved in the regulation of *ac-sc*.

## Materials and methods

### Fly strains

Fly stocks were grown on standard corn–yeast–agar medium and kept at 25°C.

The *ru h th st cu sr e ca* (*ru cu ca*) chromosome was obtained from the department of Genetics, Cambridge University. The lines *In(1)ac<sup>3</sup>*, *In(1)sc<sup>4</sup>*, *P(ry<sup>+</sup>, Delta(2–3)99B, tara<sup>1</sup>, CSN5, E7439, Df(3R)sbd<sup>26</sup>, Df(3R)P115, put<sup>P1</sup>* and *tkv<sup>1</sup>* were obtained from the Bloomington stock centre. The *pannier-Gal4*

(MD237) and *DC-lacZ* lines are described in Garcia-Garcia et al. (1999). The line carrying the SOP-enhancer and *EE4, lacZ* reporter constructs are described in Culi and Modolell (1998). The line P(SUPor-P)KG08729 was obtained from the *Drosophila* Genome Disruption Project. The strain NP-6066 was obtained from The NIG stock centre, Japan (Hayashi et al., 2002) and contains a PGawB type P-element (Brand and Perrimon, 1993). For other mutants see FlyBase (<http://flyserver.gen.cam.ac.uk:7081/>).

In order to make clones mutant for *pad<sup>1</sup>*, a stock carrying *HSFLP22; (FRT82B ry<sup>+</sup>) ry<sup>-</sup> pad<sup>1</sup>*, was crossed to *(FRT82B ry<sup>+</sup>) Ki Sb*. Second instar larvae were subjected to a 30 min heatshock.

### Isolation of the stock and mapping

The standard Balancer chromosomes, *FM7c*, *CyO* and *TM3 Sb* were used for the extraction of the chromosomes of a fly from the stock with ectopic bristles and the construction of the line A10. We used meiotic recombination to map the mutation, first with the multiple marked third chromosome *rucuca*, and later, with several *Pw<sup>+</sup>* third chromosomes (see text for details). For the SNP mapping, we proceeded as in (Martin et al., 2001) using fragments of roughly 1.5 kb that were amplified by PCR and sequenced. Polymorphic restriction sites were identified and used with the appropriate restriction enzyme (5 µl of PCR in 20 µl final volume digestion mix).

### Generation of new *poils-au-dos* alleles and mutants in the *achaete-scute* complex

We generated new alleles of *poils-au-dos* by mobilizing a P-element inserted 400 bp upstream of the predicted ATG in the line P(SUPor-P)KG08729 generated by the *Drosophila* Genome Project. New alleles of the *ac-sc* complex were created by mobilizing the P element inserted in line NP-6066 (Hayashi et al., 2002). The *P(ry<sup>+</sup>, Delta2-3)99B* third chromosome was used as a source of transposase. We recovered the hypomorphic *scute* mutant *sc<sup>bald</sup>* and several deletion mutants, including *Df(1)91B* and *Df(1)115* (see text). The deletions generated were analyzed by PCR.

### Immunostaining and in situ hybridization

Third instar larvae or white pupae were dissected in PBS and fixed for 15 min in 4% paraformaldehyde in PBT and rinsed three times for 5 min in PBT. They were incubated for 10 min in blocking solution made of 5% normal goat serum in PBT. Antibodies were used as previously described (Usui et al., 2004). They were: primary antibodies: rabbit anti-Galactosidase (Capel), guinea pig anti Senseless (Nolo et al., 2000), mouse-anti-Achaete; secondary antibodies: donkey anti-guinea pig biotin conjugated (1/200) or goat anti-mouse biotin conjugated (1/200) used in combination with streptavidin Cy3 conjugated, anti-rabbit-oregon green (1/200). Wing imaginal discs were mounted in fluoromount.

Whole mount in situ hybridization was performed using standard techniques with a *scute*-specific DIG probe. NBT/BCIP was used as a substrate for the AP staining reaction.

## Results

### Isolation of the line A10

Flies were collected in the garden of Marrakech University in 1999 (Chakir et al., 2002). A population founded by more than 30 females was selected in bulk for an increased number of thoracic macrochaetes at 17°C for the first few generations and at 25°C later. We extracted chromosomes from one female *Drosophila* with a high number (16) of ectopic bristles using balancer chromosomes. We observed that all the variation is due to the third chromosome. An isogenic line, A10, with the X and second chromosome from a wild-type stock (Oregon R)

and the third chromosome from this female was used for the following analysis. This homozygous line is perfectly viable and fertile. The phenotype is recessive: homozygotes have a marked bristle phenotype (Fig. 1B). At 25°C, females show 13.0 ( $\pm 2.28$ ) and males 9.38 ( $\pm 2.55$ ) ectopic bristles. Ectopic bristles are mainly located in the dorso-central (DC) and presutural (PS) regions. The anterior scutellar (aSC), posterior post-alar (pPA) and posterior supra-alar bristles (pSA) are also frequently duplicated in females. Additional bristles are found laterally but less frequently. These are usually slightly shorter and thicker (Fig. 1B, asterisk). The density of microchaetes is also increased. There are often four or five sensilla campaniformia on the third vein (L3) of the wing (average 3.45,  $n = 22$ ) instead of three, and at the location of the twin sensilla of the anterior wing margin (TSM), there are often three sensilla (average 2.36,  $n = 22$ ) (Figs. 1H–K).

#### Localization of the gene

We used the multiply marked third chromosome *ru cu ca* for recombination mapping and identified a single segment between *curled* (86D) and *stripe* (90E) that is responsible for the phenotype. We then employed single nucleotide polymorphism (SNP) mapping, using 30 chromosomes with a break point between *curled* and *striped* and refined the location to region 88C–89E. A study of deficiencies showed that *Df(3R)sbd<sup>26</sup>* (89B9–10; 89C7–D1) and *Df(3R)P115* (89B13;

89E7) do not complement *A10* for the bristle phenotype (Figs. 1C, D). The phenotype is thus due to one (or several) loss of function mutations in gene(s) located in the region common to both deletions: 89B13–89D1. There are about 40 genes in this region which spans around 200 kb. In order to map the mutation(s) more precisely, we made new recombinants. To select the potentially informative ones, we used P insertions with a *w<sup>+</sup>* marker located on the left (line MD237 (*pnr-Gal4*) and *tara<sup>1</sup>*) or on the right (insertion in *CSN5* and line E7439) of the mutation(s). Females *w/w*; *Pw<sup>+</sup>/A10* were crossed with males *w*; *A10/A10*. We screened >5000 flies for each P insertion and selected flies *Pw<sup>+</sup>A10/A10* and *+/A10*. We mapped the recombination point by SNP analysis in these heterozygous flies. Polymorphic sites located in the gene *sulf1* (SF4, *MspI*), between *sulf1* and *CG6901* (ST1, *NdeI*), between *CG17930* and *SF2* (CSF, *BalI*), between *CG10817* and *ss* (SS3, *DraI*) and between *ss* and *CG31279* (SS5, *SspI*) were identified and used. The mutation(s) were localized between CSF and SS3. This segment is 36.3 kb long and contains eight genes, none of which had previously been shown to have a role in bristle development. One of them, *CG10309*, encoding a zinc-finger transcription factor, had been identified in a differential screen for genes highly expressed in the embryonic nervous system (Brody et al., 2002).

We sequenced 4289 bp encompassing the *CG10309* gene in *A10*. The sequence is identical to the allele of *CG10309* in the publicly available sequence of *D. melanogaster* genome except

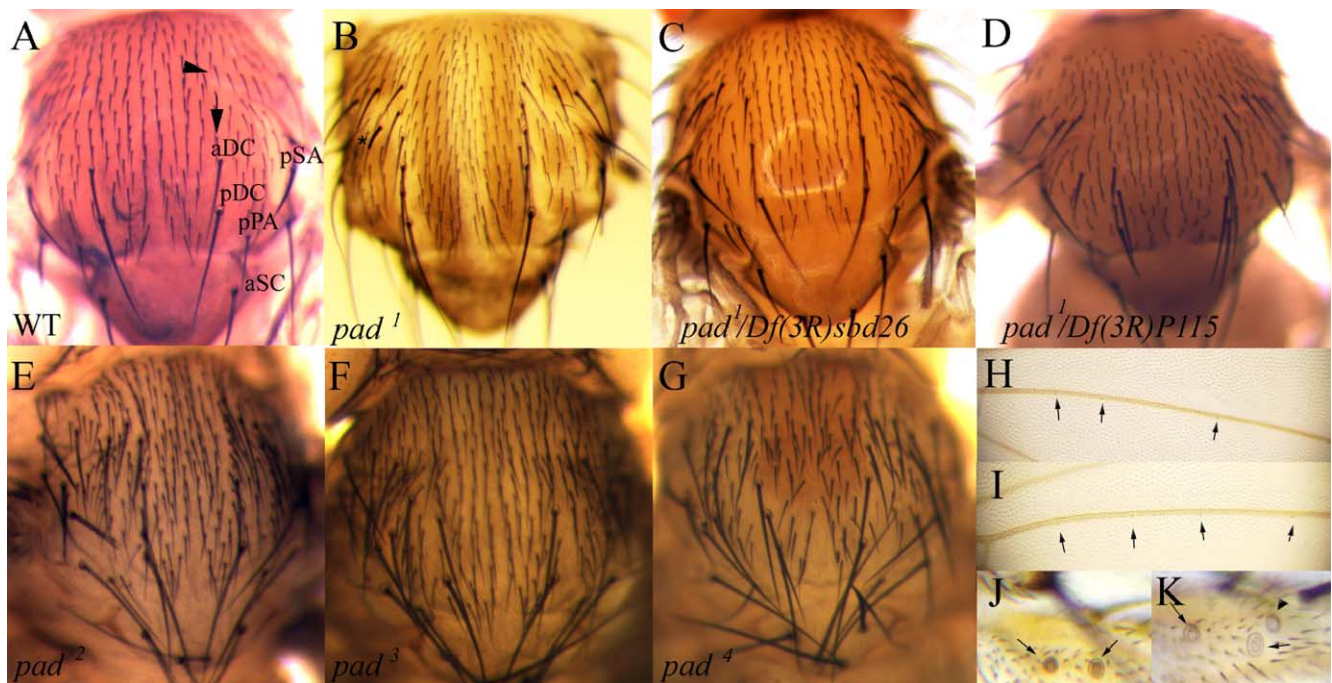


Fig. 1. Bristle phenotype of *poils au dos* mutants. Bristles on the dorsal notum are shown. (A) Wild-type flies have two dorso-central macrochaetes per heminotum (arrow). The presutural bristles (arrowhead) are slightly longer than the surrounding microchaetes. (B) In flies homozygous for the *pad<sup>1</sup>*, the number of dorso-central macrochaetes is increased, and the presutural bristles are bigger than wild type and are the size of macrochaetes. Some ectopic bristles are short and thick (asterisk). Transheterozygous *pad<sup>1</sup>/Df(3R)sbd<sup>26</sup>* and *pad<sup>1</sup>/Df(3R)P115* flies also display this phenotype (C, D). The bristle phenotype of pupal thoraces of pharate individuals carrying the new *pad* alleles, *pad<sup>2</sup>*, *pad<sup>3</sup>* and *pad<sup>4</sup>* and showing phenotypes more extreme than *pad<sup>1</sup>* are shown in panels E, F and G, respectively. Panels H and I indicate the positions (arrows) of sensilla campaniformia on the third wing vein in wild-type (H) and *pad<sup>1</sup>* mutant flies (I). Panels J and K show sensilla campaniformia (arrows) on the anterior wing margin of wild-type (J) and *pad<sup>1</sup>* mutant (K) flies. An ectopic sensilla (arrowhead) is seen in panel K. Abbreviations: aDC, anterior dorso-central; pDC, posterior dorso-central; pPA, posterior post-alar; aSC, anterior scutellar; pSA, posterior supra-alar.



for a deletion of 29 bp from position 976 to 1004 (inclusive) downstream of the A of the predicted ATG (Fig. 2A). A cDNA recently sequenced by the Berkeley *Drosophila* Genome Project (clone IP01015p; AccessionBTO22205.1) corresponds exactly to the predicted mRNA. The deletion, in the third exon, induces a frameshift and introduces 20 new codons followed by a stop codon. The resulting truncated protein is thus predicted to be 308 aa long instead of 925 aa and would lack the four zinc fingers located in the C-terminal part. We have named this gene *poils-au-dos* (*pad*) for “hairy back” in French and from now on refer to *A10* as *pad*<sup>1</sup>.

Using Blast, we identified orthologues of *CG10309* in *Drosophila pseudoobscura* and *Anopheles gambiae* (Fig. 2B). The protein sequences are extremely conserved between the two *Drosophila* species (not shown). Two well conserved domains between *D. melanogaster* and *A. gambiae* are discernable in the N-terminal and C-terminal regions (Fig. 2B). The conserved domain in the C-terminal region corresponds to four C2H2 zinc fingers likely to be involved in DNA binding. The conserved domain in the N-terminal region has recently been identified as a zinc-finger-associated domain, ZAD (Chung et al., 2002). The ZAD has so far been found only in insects and is apparently a dimerization domain involved in protein interactions (Chung et al., 2002; Jauch et al., 2003). In the *pad*<sup>1</sup> mutant, the ZAD is present but the DNA binding domain is predicted to be missing.

#### Generation of new alleles of *poils-au-dos*

In order to verify that the *pad* phenotype is indeed caused by the mutation in the gene *CG10309*, and that no other linked mutation contributes to the phenotype, we performed complementation tests. As no other mutants of *CG10309* were available, new alleles were generated by mobilizing a P-element inserted 400 bp from the predicted ATG in the line P(SUPor-P)KG08729 created by the *Drosophila* Genome Disruption project (Fig. 2A). Three independent mutant lines were recovered that failed to complement *pad*<sup>1</sup> for the bristle phenotype. All three mutants delete a large 5' portion of *CG10309* transcription unit including the region encoding the ZAD domain (Fig. 2B). The mutants were named *pad*<sup>2</sup>, *pad*<sup>3</sup> and *pad*<sup>4</sup>. All three are late pupal lethals, with a few escapers in *pad*<sup>2</sup>. In *pad*<sup>3</sup> and *pad*<sup>4</sup>, the transcription unit of the neighboring gene *SF2* is also disrupted, which correlates with the higher lethality of these mutants compared to *pad*<sup>2</sup>. To ascertain whether *SF2* is affected in *pad*<sup>1</sup>, we sequenced the whole coding frame of *SF2* in the chromosome carrying *pad*<sup>1</sup> and found no differences in the DNA sequence with the published genome sequence. The three new *pad* alleles have a more extreme bristle phenotype than that of *pad*<sup>1</sup> (Figs. 1E–G) and, unlike *pad*<sup>1</sup> flies, they also have twisted legs (not shown).

#### Expression pattern of *poils au dos*

Using in situ hybridization, we examined the expression pattern of *pad* in embryos and third instar larval wing discs. In embryos, transcripts accumulate in the central nervous system:

staining can be clearly detected above background levels shortly before stage 16 (Figs. 3A, B). This is consistent with the findings of Brody et al. (2002). No staining could be detected in the larval peripheral nervous system. We were also unable to detect staining in the wing discs. This may reflect low levels of ubiquitously expressed transcripts. We nevertheless believe that *pad* is expressed in the wing disc since *pad*<sup>1</sup> mutant clones autonomously display ectopic bristles on the notum (Fig. 3C).

#### Ectopic expression of *achaete–scute* in *poils au dos* mutants

In order to visualize the precursors of the ectopic bristles in *pad*<sup>1</sup>, we used an antibody against Senseless, a marker of neural precursors (Nolo et al., 2000) and a transgene driving the expression of LacZ under the control of the Sensory Organ Precursor enhancer (SOP-lacZ). The minimal SOP enhancer of 500 bp drives expression of *lacZ* exclusively in the bristle precursors and contains binding sites for Ac–Sc/Da (E boxes), as well as sites for the binding of repressors (Culi and Modolell, 1998). We observed that the precursors of ectopic bristles appear between 0 and 2 h after puparium formation (Figs. 4A, B). This is about the same time as the formation of the precursors for the anterior DC (aDC) bristles in wild-type flies (Huang et al., 1991). The posterior DC (pDC) precursors appear much earlier, around 24 to 12 h before puparium formation. In situ hybridization with a probe to *sc*, indicated that *sc* is expressed ectopically in third instar wing discs (Figs. 4C–F). Expression of *ac* was examined using an anti-Achaete antibody and is also significantly up-regulated in *pad*<sup>1</sup> (Figs. 4G–H). In both cases, the proneural clusters that give rise to the wild-type bristle precursors are clearly visible at wild-type locations, but they appear to be enlarged. In addition, many more cells express high levels of *ac–sc* outside the proneural clusters (Figs. 4G–H). These are mainly located in the future anterior and central regions of the notum, consistent with the fact that ectopic macrochaetes are found here (Fig. 1). Weak *sc* expression can be detected in these areas in wild-type discs but does not give rise to sense organs. Ectopic expression in *pad*<sup>1</sup> is particularly visible in the region of the presutural, DC and PSA bristles where many ectopic bristles form (Figs. 4D, F, H, arrowheads).

To better visualize the regions of ectopic expression, we used the reporter construct EE4 (Culi and Modolell, 1998) containing an artificial SOP enhancer composed of four E-boxes and the binding sites for the Ac and Sc proteins. The EE4 construct lacks the sequences required for repression and so it is very sensitive to the levels of Ac–Sc and can be used to measure the increased amounts of Ac–Sc in the *pad* mutant. We observed that expression driven by this enhancer in *pad*<sup>1</sup> is significantly different from that seen in the wild type (Figs. 4I, J). In the wild type, it is expressed exclusively in the cells of the proneural clusters where it is present at high levels (Fig. 4I) as previously described (Culi and Modolell, 1998). In *pad*<sup>1</sup>, expression in the PSA region expands medially and expression in the DC region expands anteriorly (Fig. 4J). Some of the ectopic precursors appear within this expanded anterior region

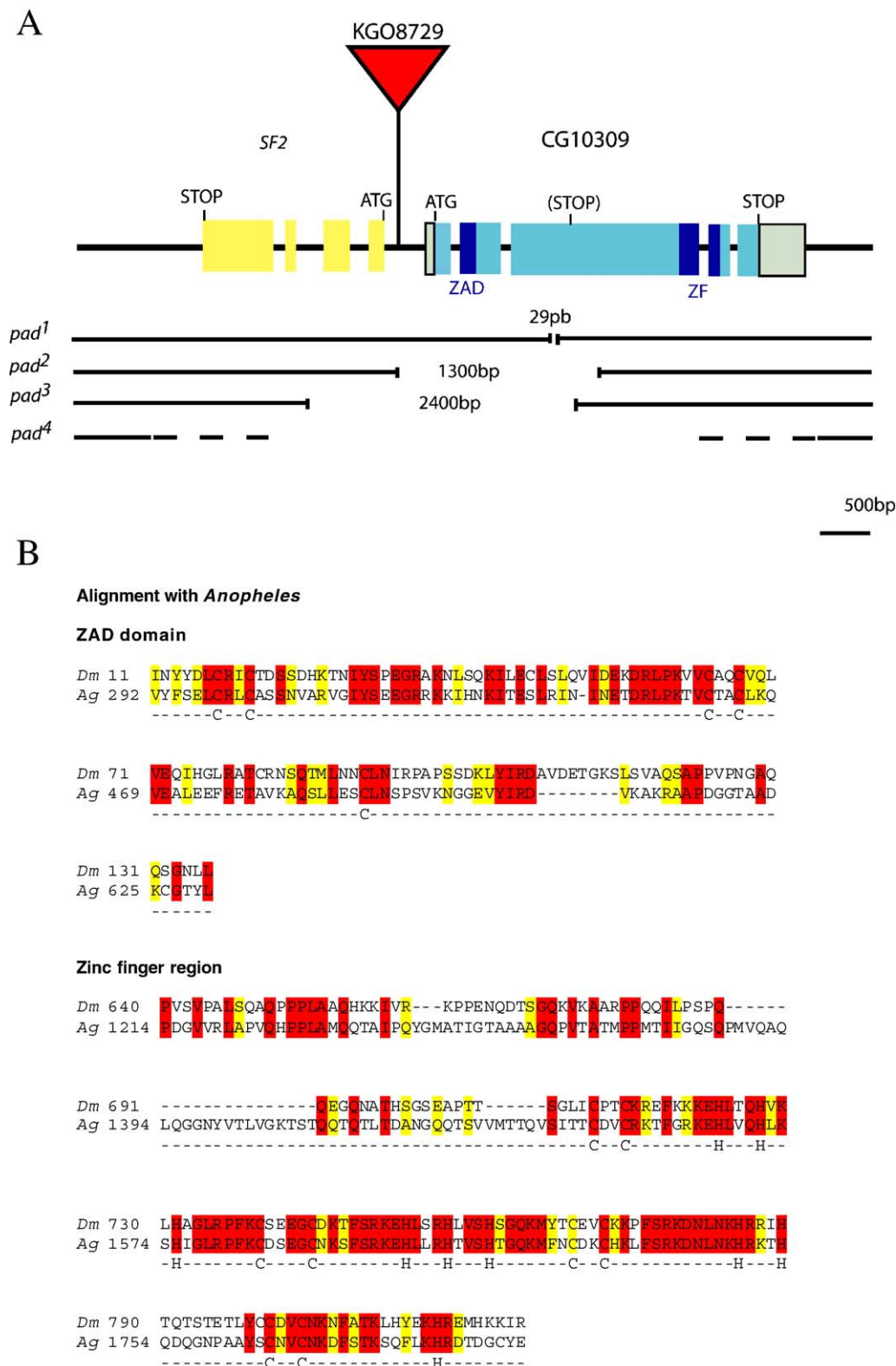


Fig. 2. Structure and sequence of the *poils au dos* gene. (A) Genomic diagram showing the coding exons for the genes *pad/CG10309* (blue boxes) and *SF2* (yellow boxes). The 5' UTR and 3' UTR of *pad/CG10309* are in grey outlined in black. The P-element KGO8729, remobilized in this study, is indicated (red triangle). The positions of the internal deletion in *pad*<sup>1</sup>, and of the deletions induced by the excision of KGO8729, are shown below. (B) Alignment of the ZAD (top) and zinc finger (middle) domains of the *pad* genes of *Drosophila melanogaster* and *Anopheles gambiae*. Identical residues are in red, conservative changes in yellow. Cysteines and histidines structurally involved in the ZAD and zinc fingers are indicated.

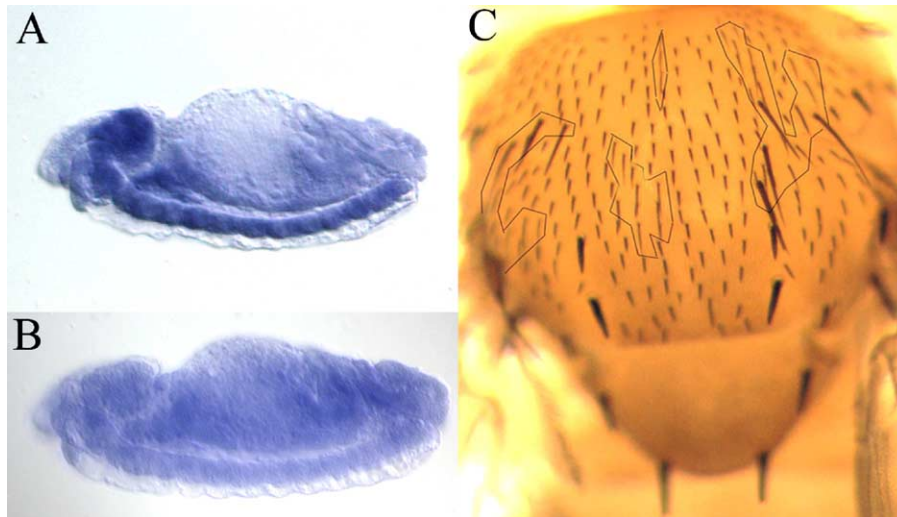


Fig. 3. Expression of *poils au dos* in embryos. In situ hybridization using an anti-sense probe to *pad* reveals accumulation in the central nervous system of stage 16 embryos (A), not seen in control embryos with a sense probe (B). Clones of cells mutant for *pad*<sup>1</sup> are outlined in panel C. The clones were labeled with *Ki*<sup>+</sup> *Sb*<sup>+</sup> and so morphology of the bristles appears normal (long shafts) among non-mutant short *Ki Sb* bristles. The clones can be seen to include ectopic bristles (cf, Fig. 1A).

(Fig. 4J, arrow). An ectopic precursor expressing *EE4-lacZ* but not yet Sens can be seen in Fig. 4J (arrowhead).

*Target sequences required for poils au dos activity are close to the scute promoter*

Expression of *ac-sc* in proneural clusters is regulated by independently-acting *cis*-regulatory enhancers (Gomez-Skarmeta et al., 1995). The enhancer responsible for activation of *ac-sc* in the cluster giving rise to the DC bristles has been characterized in detail (Garcia-Garcia et al., 1999; Romain et al., 2000). We looked at the activity of this enhancer in a reporter construct with *lacZ* (Garcia-Garcia et al., 1999). The activity of this enhancer is modified in *pad*<sup>1</sup>. The domain of expression of *lacZ* appears wider. At the same time, the anterior limit of the cluster is retracted in a posterior direction. It is possible that this is in part due to the slight distortion of the overall shape of the notum seen in *pad*<sup>1</sup> mutants. Interestingly, the ectopic bristles do not arise within the misshapen proneural cluster. They are therefore formed independently of the activity of the DC enhancer used for activation. In fact, the aDC, as well as the ectopic DC precursors, is clearly situated outside the DC cluster (Figs. 4K, L and see Discussion). We have also examined another characterized enhancer of *ac-sc*, the L3-TSM enhancer involved in the formation of the sensilla on the anterior wing margin, anterior cross vein and third vein (Gomez-Skarmeta et al., 1995) and observed no significant modification (Figs. 4M, N). These results suggest that *poils au dos* does not act through the *cis*-regulatory sequences controlling expression in the proneural clusters.

To determine which regions of the AS-C are required for the formation of the ectopic bristles in *pad*, we placed the *pad*<sup>1</sup> mutant in various *ac-sc* mutant backgrounds. These included several deletions generated by excision of the P-element in the line NP-6066, in our laboratory. We employed *In(1)ac*<sup>3</sup>, an inversion separating sequences located 1 kb upstream of *ac*,

including the DC enhancer (Campuzano et al., 1985; Gomez-Skarmeta et al., 1995), *Df(1)91B*, which deletes 45 kb from a position 10.3 kb upstream of *sc* that includes *ac* and the DC enhancer, *Df(1)115* which deletes 7.8 kb between the positions 14.5 and 6.7 kb upstream of the *scute* ATG, and *In(1)sc*<sup>4</sup>, an inversion with a breakpoint 7–8 kb downstream of *sc* (Campuzano et al., 1985) (Fig. 5). None of these rearrangements prevent formation of the ectopic bristles present in *pad*<sup>1</sup> (Figs. 6A–H). *In(1)sc*<sup>4</sup> causes a loss of all scutellar bristles, because the relevant enhancer, located 40 kb downstream of *sc*, is translocated elsewhere and is thus not able to drive the expression of *ac-sc* in the scutellum (Gomez-Skarmeta et al., 1995). However, occasional scutellar bristles form in *In(1)sc*<sup>4</sup>; *pad*<sup>1</sup> flies at the position normally occupied by the anterior scutellar bristle (Figs. 6G, H). In contrast to the rearrangements cited above, no, or very few, ectopic bristles are formed in *sc*<sup>bald</sup>; *pad*<sup>1</sup> flies (Figs. 6I, J). This hypomorphic *sc* allele carries the remains of a P element located 10 kb upstream of *sc* (Fig. 5) and displays a high frequency of missing SC, aDC and orbital bristles. Together, these results indicate that the target sequences are probably located in a fragment that extends 6.7 kb upstream and 7–8 kb downstream of *sc* (Fig. 5).

*poils au dos interacts genetically with other repressors of achaete-scute*

The generalized increase in *ac-sc* expression suggests that *poils au dos* is involved in the repression of *ac-sc*. We therefore tested interactions between *pad* and other known repressors of *ac-sc*. We found that *pad*<sup>1</sup> interacts moderately with *emc*<sup>pel</sup> (not shown) and very strongly with *hairy*<sup>1</sup>. In *h*<sup>1</sup> homozygotes grown at 18°C, ectopic bristles are occasionally found anterior to the aDC (Fig. 7A), whereas none is seen at 25°C. In *h*<sup>1</sup> *pad*<sup>1</sup> homozygotes, many ectopic bristles are observed at 25°C at positions where none are seen in either of the single mutants. These include DC bristles closer to the



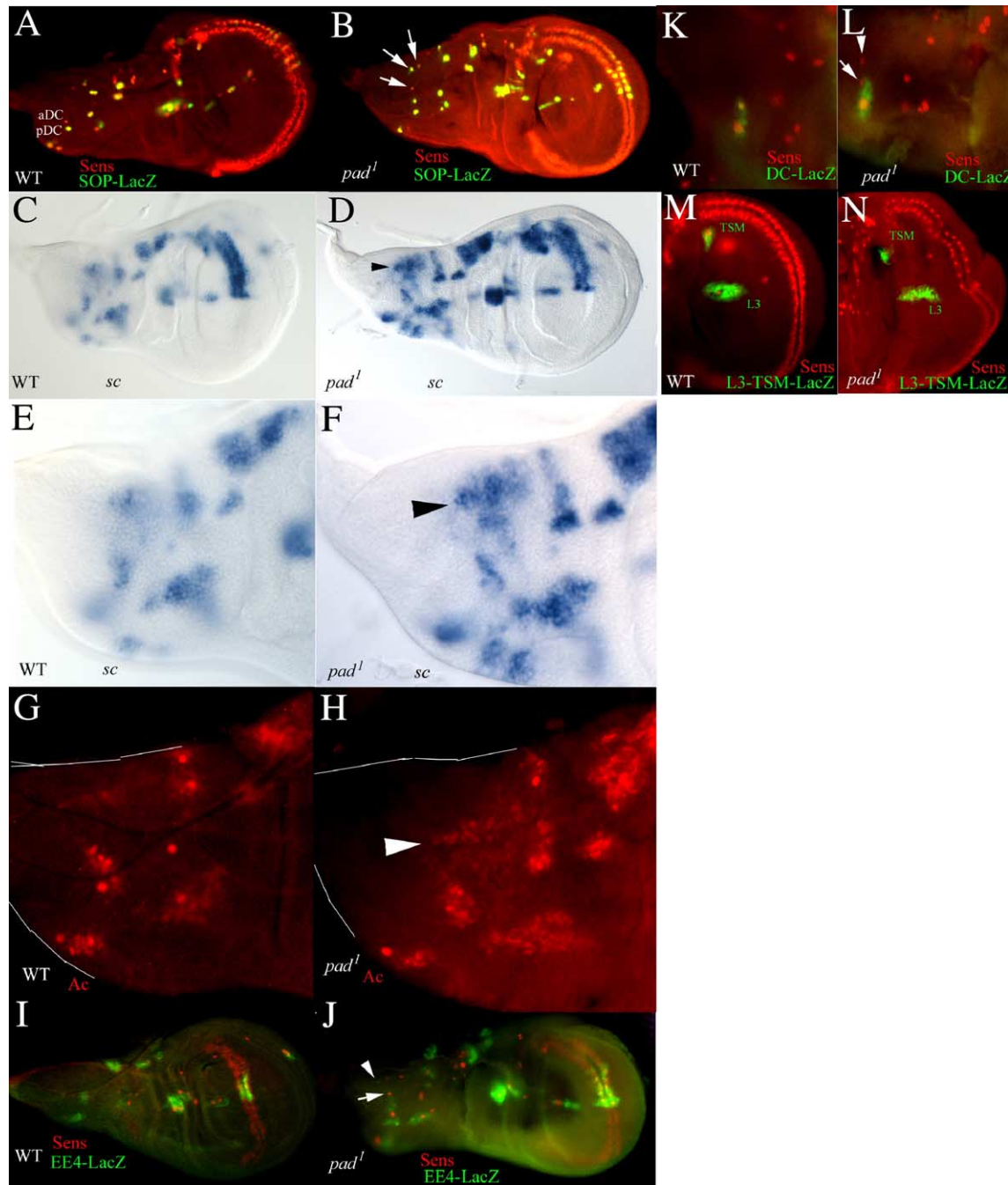


Fig. 4. Visualization of ectopic bristle precursors and *achaete-scute* expression in *poils au dos* mutants. Anti-Senseless staining and expression of a LacZ reporter construct for the SOP enhancer activity of wing discs collected from wild-type (A) and *pad<sup>1</sup>* (B) individuals at 2h after puparium formation. Senseless (red) and LacZ (green) indicate the positions of bristle precursors. Additional dorso-central and presutural precursors are seen in the mutant (arrows). The results of in situ hybridization with a probe to *scute* are shown in wild-type (C, E) and *pad<sup>1</sup>* mutant (D, F) third instar larval wing discs. *scute* is ectopically expressed in *pad<sup>1</sup>*, especially in the presutural (arrowhead), DC and PSA regions. Staining with an anti-Achaete antibody shows the positions of the proneural clusters in wild-type (G) and *pad<sup>1</sup>* (H) discs. Higher levels of basal Ac are also seen in the lateral region of the notum of mutant discs. Panels I and J show anti-Senseless staining (precursors, red) together with β-galactosidase (green) used as a reporter for expression of an artificial enhancer composed of only four E-boxes (EE4). This is expressed in proneural clusters 1 h after puparium formation in the wild type (I), but can be detected in broader, more continuous domains in *pad<sup>1</sup>* mutant wing discs (J). Bristle precursors are more numerous in the mutant. Note that an ectopic precursor is situated in the expanded domain of EE4 enhancer-driven expression (arrow). An ectopic precursor expressing the EE4-lacZ but not yet *sens*, is visible (arrowhead). Panels K and L show anti-Senseless staining (red) together with β-galactosidase (green) used as a reporter for expression of the DC enhancer sequence that drives expression in the dorso-central proneural cluster. The anterior dorso-central bristle (aDC), inside the cluster in the wild type (K), is outside the cluster in mutant *pad<sup>1</sup>* wing discs (L, arrow). An ectopic dorso-central precursor is indicated by the arrowhead in panel L. Panels M and N show the wing pouch with double staining for the L3-TSM lacZ enhancer reporter (green) and Senseless (red) in wild-type (M) and in a *pad<sup>1</sup>* mutant fly (N). The activity of the enhancer is changed little in the mutant.

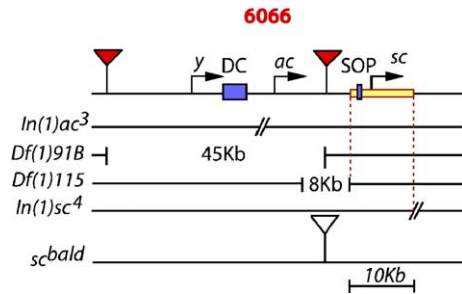


Fig. 5. Location of deletions and inversion breakpoints at the AS-C used in this study. The inversion breakpoints of *In(1)ac<sup>3</sup>* and *In(1)sc<sup>4</sup>* are indicated by diagonal double bars. The deletions *Df(1)91B* and *Df(1)115*, as well as the regulatory mutant *sc<sup>bald</sup>*, were generated by mobilizing the P elements in the line NP-6066 that carries the two *Pw<sup>+</sup>* insertions indicated by the red triangles. The region containing the sequences necessary for the formation of the ectopic bristles in *pad<sup>1</sup>* is in yellow, delimited by red lines.

thoracic midline and additional bristles between the anterior and posterior scutellars (Fig. 7B). Interestingly, most of these ectopic bristles are located in the posterior half of the notum whereas the visible effect of *pad* alone is in the anterior part of the notum.

Mutations in very few other genes have been shown to induce ectopic bristles in the anterior region of the notum. Some ectopic bristles can be induced in this region by reduction in Dpp signaling late in development (Phillips et al., 1999). We tested for a genetic interaction between *pad* and Dpp signaling using mutations in the receptors *punt* (*put*) and *thickveins* (*tkv*). We observed a strong genetic interaction between *pad<sup>1</sup>* and *put<sup>P1</sup>*. Trans-heterozygous *put<sup>P1</sup>/pad<sup>1</sup>* flies have ectopic DC bristles (Fig. 7C) whereas each of the single heterozygotes displays a wild-type pattern (not shown). Flies homozygous for the hypomorphic mutation *tkv<sup>1</sup>* occasionally have ectopic bristles anterior to the aDC at 18°C (Fig. 7D). The phenotype is strongly enhanced in the anterior region of the notum of double mutant *tkv<sup>1</sup>; pad<sup>1</sup>* flies grown at 25°C. In particular, many more ectopic bristles are visible around the prescutal suture (Fig. 7E) than in *pad<sup>1</sup>* alone (Fig. 1B).

## Discussion

*The phenotype of the line selected for an increased number of dorso-central bristles is due to a single locus*

An important question in evolutionary biology is to understand the relationship between intraspecific variability in morphological traits and their interspecific divergence (Nuzhdin and Reiwitz, 2000). Selection experiments can help to answer this question, since they allow identification of the genetic variation relevant to a specific trait present in the

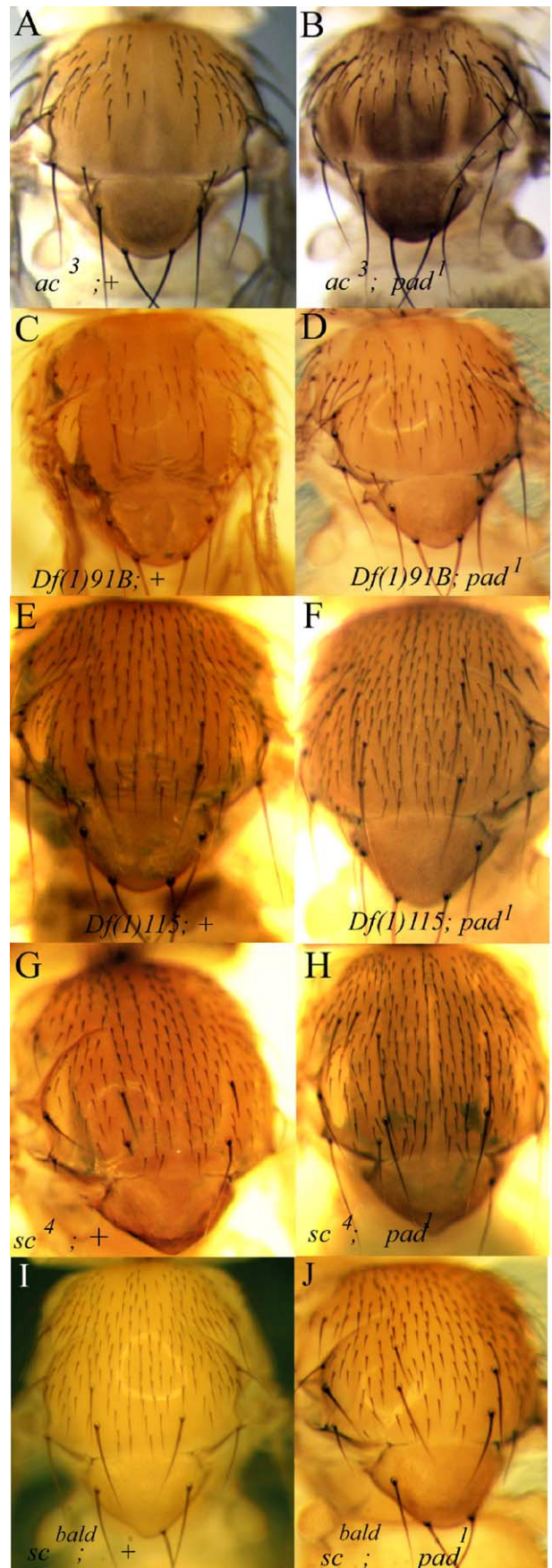


Fig. 6. Bristle phenotype of flies mutant for *poils au dos* and carrying rearrangements of the AS-C. Thoraxes are from flies whose third chromosomes are either wild type (left column: A, C, E, G, I) or *pad<sup>1</sup>* (right column: B, D, F, H, J), and whose first chromosomes carry *In(1)ac<sup>3</sup>* (A, B), *Df(1)91B* (C, D), *Df(1)115* (E, F), *In(1)sc<sup>4</sup>* (G, H) and the regulatory mutant *sc<sup>bald</sup>* (I, J). It can be seen that only *sc<sup>bald</sup>* reduces the number of ectopic bristles in the *pad* mutant (cf Fig. 1B).



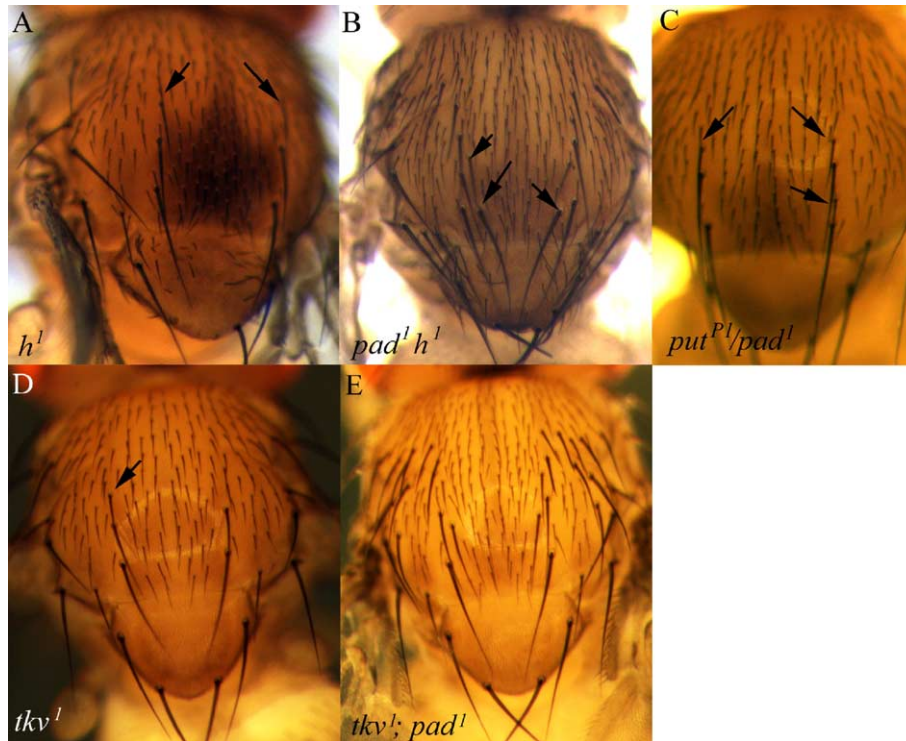


Fig. 7. Bristle phenotype of flies homozygote for *hairy*<sup>1</sup> grown at 18°C (A) and homozygote double mutant for *hairy*<sup>1</sup> *pad*<sup>1</sup> (grown at 25°C) (B) to be compared with *pad*<sup>1</sup> single mutant (Fig. 1B). Note the ectopic bristles located at positions where none are observed in either of the single mutants (Fig. 1B, arrows). Interaction between *pad* and Dpp signaling revealed by the bristle phenotype of *put*<sup>P1</sup>/*pad*<sup>1</sup> transheterozygous flies (C), *tkv*<sup>1</sup> homozygotes (D) and *tkv*<sup>1</sup>; *pad*<sup>1</sup> double homozygotes (E). Note the ectopic bristles (arrows in panels C and D). In *tkv*<sup>1</sup>; *pad*<sup>1</sup> flies, there is a general increase in the number of bristles around the sutural region.

selected population. In this study, the quick response to artificial selection has relied on the fixation of a single loss of function allele with a strong phenotypic effect. Interestingly, other examples from natural populations have shown that some important morphological variations, such as pelvic reduction in fresh-water sticklebacks can be caused by a major or even a single locus (Shapiro et al., 2004). However, previous studies on bristle QTL in selected lines of *Drosophila* have always identified several major QTLs (Gurganus et al., 1998; Long et al., 1995). In the case of the DC bristles, the three major chromosomes contributed to the response to artificial selection in a previous experiment (Dominguez et al., 1993). It is therefore unusual that the phenotype in our selected line relies on a single bristle QTL (other loci may contribute quantitatively, but the *pad*<sup>1</sup> mutation is necessary and sufficient for the phenotype). The *pad*<sup>1</sup> mutation is probably present at very low frequency in Marrakech, since a second sample of flies collected in the same location did not give a strong response to selection suggesting an absence of the mutation (unpublished). Furthermore, the phenotype appears to be due to a single deleterious lesion in the *pad* gene; many single QTLs contain multiple mutations. We do not know whether deleterious variants of this kind contribute to long term evolution but such polymorphisms need not necessarily be devoid of evolutionary advantage. Indeed, some natural situations have been described where loss of function mutations presenting a particular advantage are rapidly fixed during an adaptative radiation. Recent examples in multicellular eukaryotes include

loss of function mutations in a pigmentation gene inducing a shift of pollinators in *Petunia* (Quattrocchio et al., 1999).

The gene we have uncovered encodes a zinc-finger transcription factor with a ZAD domain. ZAD domains have only been described in insects where they are highly represented: there are about 80 of them in the *Drosophila* genome (Chung et al., 2002). Almost all are found in association with zinc finger domains. Few have been studied, but in several cases, point mutations in the ZAD domain have been shown to completely disrupt the function of the gene (Croizatier et al., 1992; Gaszner et al., 1999). The ZAD domain of the Grauzone transcription factor has recently been crystallized and shown to be a dimerization domain (Jauch et al., 2003). The global structure of the ZAD domains is remarkably conserved and so it is possible that different transcription factors are able to form heterodimers through their ZAD domains. The ZAD domain encoded in *pad* might therefore have a crucial role in establishing molecular interactions between different transcription factors.

In addition to the bristle phenotype, the alleles *pad*<sup>2</sup>, *pad*<sup>3</sup>, and *pad*<sup>4</sup> die as late pupae with twisted legs, a phenotype caused by abnormal eversion of leg imaginal discs during pupal development. Unlike these alleles which are predicted not to encode any protein, *pad*<sup>1</sup> with a predicted truncated protein does not display this phenotype. A similar phenotype is seen in mutants of the gene *crooked-legs* which encodes a zinc-finger transcription factor showing significant similarity to Pad in the zinc fingers (D'Avino and Thummel, 1998). It is the

most closely related gene to *pad* in the *D. melanogaster* genome according to Blast analysis.

*poils-au-dos* is a newly identified gene involved in the regulation of *achaete–scute*

We show that *pad* is involved in the regulation of *ac–sc*. In *pad* mutants, expression of *ac* and *sc* is increased. The increase appears to be general in that the proneural clusters are enlarged, but in addition, other cells with high levels of Ac–Sc are seen in regions where these products are normally absent or barely detectable. This phenotype is reminiscent of the *Hw* mutations that are associated with a generalized increase in the levels of Ac and Sc (Balcels et al., 1988). Enlargement of the proneural clusters need not necessarily result from increased activity of the *cis*-regulatory sequences that normally drive them but can be seen as a consequence of a ubiquitous increase in gene expression. Indeed, our results suggest that ectopic bristles do not arise from proneural clusters. Use of the DC enhancer with a *lacZ* reporter showed that the ectopic bristles are outside the area of activity of this enhancer. Furthermore, DC bristles also form in the absence of this regulatory sequence (*In(1)ac<sup>3</sup>* and *Df(1)91B*). In wild-type flies, the precursors for the pDC and then later the aDC arise from the DC cluster. In *pad* mutants, a bristle immediately anterior to the pDC, the “aDC”, is outside the area of *lacZ* staining. This suggests that a bristle at this position, forms earlier in *pad* mutants from cells with a high level of *ac–sc* expression not driven by the endogenous enhancer. The presence of such a bristle would then prevent the formation of a precursor from the DC-driven cluster itself, by means of Notch-mediated inhibition. Scutellar bristles can also form in the absence of the scutellar enhancer (*In(1)sc<sup>4</sup>*) and a reporter construct for the L3-TSM enhancer (Gomez-Skarmeta et al., 1995) is unchanged in *pad<sup>1</sup>*. Therefore, *pad* is unlikely to act via each of the enhancer modules that mediate activation in proneural clusters (Gomez-Skarmeta et al., 1995). It is more probable that Pad acts as a repressor to prevent generalized accumulation of Ac–Sc over the notum and in particular outside the sites of the proneural clusters. The strong genetic interaction between *pad* and *hairy*, a known transcriptional repressor of *ac–sc*, as well as the synergism with Dpp signaling is in agreement with this hypothesis.

We have defined a 13 kb region round *sc* that is likely to contain sequences necessary for the formation of all ectopic bristles in *pad* mutants. We hypothesize that these sequences direct a weak expression of *ac–sc* over the entire notum that is up-regulated in *pad* mutants. One possibility is that Pad acts on the *sc* promoter. Within the region delimited above, a 3.7 kb stretch upstream of the *scute* ATG, containing both the SOP enhancer and an enhancer for the wing L3-TSM region, is expressed in several proneural clusters and some other regions (Martinez and Modolell, 1991). In the absence of Ac and Sc, expression, albeit weak, of a 3.7 kb-*lacZ* reporter construct can still be detected in the regions of the DC and PSA bristles. Therefore, this fragment drives expression of *sc* prior to the

onset of autoregulation (Martinez and Modolell, 1991). *pad* may act, directly or indirectly, via these sequences, allowing a level of Sc high enough in some cells for autoamplification and the adoption of a neural fate. This is visible in cells where the *EE4-lacZ* is activated, prior to expression of *sens* and activation of the SOP enhancer. Further studies are required to determine the mechanism of Pad function and whether these sequences are indeed a target for Pad.

*The use of natural variation to identify new genes regulating development*

Variation of bristle number in *Drosophila* has been a classical model for quantitative genetics. Traditionally, the phenotype of interest is generated by selection followed by a search for the genetic factors responsible. Several QTLs causing variation in bristle number have been identified in this fashion (Gurganus et al., 1998; Long et al., 1995). However, their resolution at the single gene level has only been successful when candidate genes known to affect bristle development were found to map within the QTLs, as confirmed by the more detailed study of some of them (Long et al., 1998, 2000; Lyman et al., 1999; Lyman and Mackay, 1998). This is also true for studies on other models (Shapiro et al., 2004). In fact, there are very few examples where a new, previously unknown gene, has been shown to be responsible for a QTL. This can be attributed to the difficulty of obtaining enough informative recombinants within the QTL. Indeed, one of the rare successful examples corresponds to a QTL located in a recombination hot spot (Fridman et al., 2000). Furthermore, most quantitative traits are influenced by several QTL, which makes the mapping more difficult than in this study. The intensive focus on *D. melanogaster* as a genetic model has led to the development of a number of tools that allow the efficient mapping of mutations and the rapid, precise resolution of QTLs. The genome sequence now provides access to all genes and allows the sites of recombination to be mapped using SNPs (Martin et al., 2001). Furthermore, the generation of thousands of precisely located transposon insertions labeled with convenient markers such as *white<sup>+</sup>*, means that informative recombinants can be efficiently identified (Bellen et al., 2004; Zhai et al., 2003). Indeed, the use of *Pw<sup>+</sup>* insertions located at proximity to the QTL significantly reduces the effort involved in SNP mapping, and also the cost since the proportion of uninformative recombinants is much lower. In addition to quantitative variation of bristles, morphometric traits such as body weight, wing and thorax length, ovariole number or pigmentation, vary significantly between wild populations which have adapted locally (Gibert et al. 2004). These too could be amenable to studies allowing the identification of new regulatory genes. An advantage of natural variants is that the mutations responsible for phenotypic differences are likely to be less severe in general than the complete loss of function mutations frequently generated by traditional mutagenesis. Such hypomorphic mutations facilitate the study of adult phenotypes. Therefore, the combined use of natural variation observed in lines selected from wild popula-

tions of *Drosophila* and the powerful genetic tools provided by laboratory strains permits identification of new genes as illustrated here.

## Acknowledgments

We thank the various members of the Simpson lab for their comments on the manuscript and their interest in the project, and Claire Chapman for excellent technical assistance. We thank Juan Modolell (Madrid, Spain), the *Drosophila* Genome Disruption Project, the NIG Stock centre, Japan, the Genetics Department of Cambridge University and the Bloomington *Drosophila* stock center for fly stocks, Hugo Bellen for the Senseless antibody and the various labs who sent stocks and antibodies not mentioned in this paper. J.-M. Gibert was the recipient of a Marie Curie European fellowship. This work was supported by the Wellcome Trust.

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